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Breast Cancer Cells Induce a Pro-inflammatory Response to Mitigate Immune Mediation in a 3D Culture Model

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Abstract. *Background: Growth factors and cytokines mediate complex interactions between cells within the breast tumour microenvironment. In advanced cancer, an excess of regulatory T (T_{REG}) lymphocytes and lack of natural killer (NK) cells in tumour-infiltrating lymphocyte populations may reflect a shift to pro-tumorigenic adaptive immune mechanisms. To facilitate targeted assessment of the interactions between tumour and immune cells ex vivo, three-dimensional (3D) culture systems are able to better recapitulate the in vivo microenvironment, recreating the anatomy of tumours. Materials and Methods: We used 3D breast tumour models to determine morphological alterations, and the levels of secreted transforming growth factor-β (TGFβ) and induced cytokines. 3D luminal phenotype models and basal phenotype models were generated by culturing NK cells and $CD4^+CD25^+$ T_{REG} cells with MCF-7 cells and MDA-MB-231 cells respectively, in growth factor-reduced Matrigel. TGFβ was qualitatively assessed by immunolocalisation and cytokine data from culture supernatant was acquired with a multiplex cytokine assay. Traditional statistical analysis and principal component analysis were employed to unravel the cytokine response. Results and Conclusion: We identified that an interleukin-6 (IL6)–chemokine axis associated with TGFβ is primarily responsible for differences detected between breast cancer models, with luminal and basal phenotype tumours responding differentially to immune mediation. Identified cytokines are implicated in facilitating tumour cell subversion of immune cell function to promote an invasive phenotype. Moreover, the disruption of the extracellular matrix and failure to form well-differentiated tumour masses/networks is indicative of enhanced malignancy. Tumour cells are implicated in promoting a pro-inflammatory microenvironment to attenuate NK cell function and in inducing a pro-tumorigenic profile that is facilitated by T_{REG} lymphocytes.*

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Tumours consist of diverse cell populations, the interactions of which are essential to either facilitate tumour cell elimination or escape from immunosurveillance mechanisms (1, 2). Immunophenotyping of tumour-infiltrating lymphocytes (TILs) has revealed that generally, $CD8^+$ cytotoxic T-lymphocytes and natural killer (NK) cells are associated with anti-tumour responses (3), with variable levels of these effector cells noted in tumour subtypes (4). While NK cells primarily exert their anti-tumour function in a direct cell-mediated manner, their ability to produce an array of cytokines allowing for interaction with malignant cells and regulatory T (T_{REG}) lymphocytes is suggested to be their principal role in controlling tumour progression (5, 6).

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer-related death in females (7). Classification methods for treatment stratification are based primarily on presentation of the oestrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 (HER2), allowing identification of three major phenotypes: Luminal or hormone-dependent; HER2-overexpressing; and basal or hormone-independent tumours (8). A high TIL population is associated with better survival in patients presenting with triple-negative or $HER2^+$ breast cancer; however, its significance in cancer with the luminal phenotype remains elusive, although it is proposed that focus on specific immune cell subsets may yield more promising results (3, 4). Traditionally, breast tumours were not regarded as immunogenic; however, increasing evidence suggests a

role for TILs as a predictive indicator of survival and response to treatment (2, 9). It is further suggested that the immune cell subsets, proportionally and functionally, within the TIL population are paramount, as is the phenotype of the tumour itself (4).

In advanced breast cancer, the accumulation of T_{REG} lymphocytes, and the scarcity of NK cells in TIL populations, despite an elevated presence in the circulation (3, 10, 11), may reflect the supremacy of adaptive immune mechanisms with the concomitant inhibition of innate immunity. T_{REG} lymphocytes are typically associated with pro-tumoural responses and dominate TILs of more aggressive luminal and basal phenotype tumours (3, 12-14). T_{REG} lymphocytes are implicated in facilitating immunoediting processes by liaising with tumour cells, promoting reduced immunogenicity and actively suppressing anti-tumour functions of cytotoxic T-lymphocytes and NK cells directly, *via* cell–cell contact and indirectly, *via* cytokine mediation (5, 15, 16).

Cytokines are low molecular weight proteins that mediate inflammation by recruiting leukocytes, enabling cell–cell cross-talk and inducing signalling pathways, thereby facilitating homeostasis (6, 17). With multiple cytokines that are, moreover, pleotropic in nature, signalling is highly complex, forming networks that remain to be fully elucidated. In the tumour microenvironment, the capacity of cytokines to act in an augmentative, synergistic or antagonistic manner is subverted and these extracellular mediators ultimately induce an immune-suppressive microenvironment that facilitates tumour progression (6, 9). Key players that have been identified include interleukin-6 (IL6), a pro-inflammatory cytokine that has pleiotropic functions and is produced by an array of cell types, including immune cells, fibroblasts, antigen-presenting cells and tumour cells themselves (6, 17-19). IL6 is a critical driver of tumorigenesis, associated with heightening hormone-dependent tumour cell proliferation and epithelial–mesenchymal transition (EMT) (18, 19). As part of the type I cytokine family, IL6 exhibits a functional homology with IL2 and IL12, both of which are noted for their capacity to activate T-lymphocyte subsets and NK cells (6). Activation of NK cells in turn, elicits the production of interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα), cytokines implicit in cytolytic elimination of tumour populations (20); conversely, these cytokines are also able to induce cytokine cascades including IL1β, IL6 and TNFα which amplify pro-inflammatory processes and pro-tumour pathways (1). Additionally, chemokines including chemokine C-C motif ligands, CCL2 and CCL4, and chemokine C-X-C motif ligand CXCL8, contribute to the inflammatory microenvironment recruiting additional leukocyte subsets (21, 22). However, these chemokines also show efficacy as pro-angiogenic factors and are implicated in tissue remodelling *via* matrix metalloproteinase (MMP) induction, a process necessary for metastasis (22-24). Initiation of metastasis is also dependent on the ubiquitous molecule, transforming growth factor-β (TGFβ), which in late-stage tumours is an essential driver of EMT and invasion (25, 26); and furthermore, is implicated in T_{REG} lymphocyte-mediated suppression of NK cell function (27-29).

To unravel these cellular interactions, in previous work we described a 3D culture system incorporating NK cells, prototypic $CD4^+CD25^+$ T_{REG} lymphocytes and breast cancer cells (30). For *in vitro* investigations, 3D culture systems are more effective in reproducing the tumour microenvironment than 2D systems (19, 31, 32), and allow for more appropriate inferences to be made regarding secreted growth factors and cytokine profiles. Investigation into cytokine networks has been enhanced by the development of fluorophore-conjugated bead-based multiplex immunoassays which provide a mechanism by which a range of cytokines can be simultaneously investigated. This system allows for capturing low levels of analytes of interest in small sample volumes with a high degree of sensitivity and specificity (22). Data analysis is however, hampered by a loss of data during the conversion of fluorescence intensities to concentration data, and by low- or high-level analyte abundance, which while subject to a variety of imputation methods that may approximate abundance can also lead to loss of variables of interest (33-35). As such, traditional statistical analyses which are largely affected by data censoring may be limited in defining cytokines of biological interest; the use of exploratory analysis may assist, nevertheless, in gaining important biological information.

In this study, we investigated the response of luminal and basal phenotype breast cancer cells to immune mediation in a 3D culture system by qualitatively assessing alterations in cellular morphology and TGFβ expression, and by quantitatively assessing the cytokine response using both standard statistical techniques and exploratory principal component analysis to account for the loss of data during censoring.

Materials and Methods

Human Ethics Clearance was obtained from the Human Ethics Research Committee (Medical), University of the Witwatersrand, Clearance Certificate Number M081036 and M140155. Informed consent was obtained from all blood donors. Heterotypic 3D culture models were established as described by our laboratory (30). In brief, approximately 30 ml blood from healthy female volunteers between the ages of 18 and 35 years (exclusion criteria were pregnancy, autoimmune diseases, immunodeficiency, cancer and a previous history of cancer) was collected in EDTA-coated Vacutainers by venepuncture. Peripheral blood mononuclear cells were obtained *via* density gradient centrifugation using Ficoll-Hypaque (1.077 g/cm^3) (GE Healthcare Biosciences AB, Uppsala, Sweden). The prototypic T_{REG} lymphocyte population was isolated

using $CD4^+$ Multisort Microbead Kit (Miltenyi Biotec, Cologne, Germany) followed by subsequent isolation of $CD25^+$ cells (Miltenyi Biotec). The unlabelled fraction was collected for subsequent isolation of NK cells. NK cells were labelled with allophycocyanin (APC)-NKp46 (Miltenyi Biotec) and magnetically isolated using anti-APC microbeads (Miltenyi Biotec). The efficacy of the magnetic sorting procedure was validated using flow cytometry. The median yield of live cells and viability as assessed using the trypan blue exclusion assay and Bio-Rad Automated Cell Counter TC-20 (Bio-Rad, Hercules, CA, USA) were as follows: T_{REG} lymphocyte yield 1×10^6, viability 88.5%; NK cell yield 1.1×10^6, viability 83.2%. Following 48 h activation of T_{REG} lymphocytes and NK cells with IL2 and phytohemagglutinin (36), immune cell populations were allocated to co-culture groups.

A heterotypic luminal phenotype (LPM) culture model used the MCF-7 cell line (Sigma Aldrich, St. Louis, MO, USA) at passage number 14. The MDA-MB-231 cell line (Sigma Aldrich) at passage number 48, was used as the basal phenotype (BPM) culture. These cancer cell lines were resuspended with isolated lymphocyte subgroups at a ratio of 2:1 in RPMI 1640 culture media supplemented with 10% foetal bovine serum (Biocom Biotech, Clubview, South Africa) and 0.1% penicillin/streptomycin (Sigma-Aldrich) with growth factor-reduced Matrigel (GFRM) (BD Biosciences, Woodmead, South Africa), in duplicate. Thus the LPM, based on MCF-7 cells; and the BPM, based on MDA-MB-231 cells each consisted of the following culture groups: Experimental: the experimental culture groups containing T_{REG} lymphocytes, NK cells and breast cancer cells; NK-BC: a control culture group including only NK cells cultured with breast cancer cells; T_{REG}-BC: a control culture group including only T_{REG} lymphocytes cultured with breast cancer cells; BC: a control culture group in which breast cancer cells alone were cultured.

The cultures were incubated in the aforementioned culture media at 37˚C in 5% CO_2 for 72 h. Brightfield and fluorescent images were obtained using an Olympus iX51 inverted fluorescent microscope with CellSens Software (Wirsam Scientific & Precision Eq. Ltd, Johannesburg, South Africa) and plates generated using GIMP 2.10.12 software.

Sample collection. Supernatants from four repeated experiments were collected, snap-frozen in liquid N_2 and stored at −80˚C until cytokine analysis. For immunohistochemical analysis, the cultures were rinsed in PBS prior to incubation in 30 µl fetal bovine serum at 37˚C for 1 min. Cultures were then incubated with 30 µl thrombin (SANBS, Pretoria, South Africa) for approximately 30 s at 37˚C, then at room temperature for a further 5 min for clot formation. Samples were fixed in 200 µl 4% paraformaldehyde (Sigma-Aldrich) in PBS for 20 min, the fixative drained and eosin added to the wells for visualisation of the samples. Samples were removed from the wells with a plastic pipette and placed into a 0.45 µm filter paper envelope for automatic tissue processing. The samples were thereafter embedded in paraffin wax (Merck Millipore, Darmstadt, Germany) and stored at 4˚C until sectioning (30, 37).

Cytokine analysis. The Bio-Plex Pro cytokine assay (Bio-Rad) was used to detect the presence of the following cytokines IL1β, IL2, IL6, IL12, IFNγ, TNFα and the chemokines CCL2, CCL4 and CXCL8, in 50 µl of undiluted sample. Eight standards and two blank controls consisting of culture media, and GFRM-conditioned culture media were used. Data were acquired using the Bio-Plex 200 system and Bio-Plex Manager™ software (Bio-Rad). For cytokine analysis GFRM-conditioned culture media and culture media alone were used as blank controls. No significant differences regarding any of the variables were found between these blank controls.

For assessment of cytokine fluorescence data, data were first censored whereby the lower limit for each cytokine detected was defined as greater than that of the blank controls. The dataset which consisted of concentration values (pg/ml), in which all values reported as being out of range (OOR), be it too low or too high, were deleted; and values between the lower limit of quantitation and upper level of quantitation were retained. Any values that fell below this limit were regarded as undetectable and were deleted. Mean value imputation was used for replacing missing data values only where data points present were ≥50% within each culture group. This resulted in certain culture groups where no values were detected.

Following censoring of the dataset, for a traditional approach to assessing significant differences between the models, given the non-parametric nature of the data, the Mann–Whitney *U*-test (two-sided, $p<0.05$) (Statistica v12, Statsoft Southern Africa, Johannesburg, South Africa) was used to assess whether luminal phenotype and basal phenotype breast cancer cells differentially responded to immune mediation by altering their cytokine production within each culture group. Furthermore, the Kruskal–Wallis by ranks ANOVA, followed by a multiple comparison *post-hoc* test was used to determine the effects of immune mediation within each model.

In order to explore the data and assess the presence of associated patterns, principal component analysis [PAST v2.17c., free software (38)] was conducted on log transformed data. Principal component analysis on a variance-covariance matrix allowed the variables to be reduced into components that accounted for the majority of the variance in the dataset (35) *i.e.* the greatest variance would be accounted for by the first principal component (PC1) and the second greatest variance accounted for by the second principal component (PC2). The contribution of each individual cytokine to the derived components was assessed using factor loadings.

The equation

$$1/\sqrt{n(cytokines)-1)}$$

was used to determine the cut-off level for assessing the importance of each cytokine to the derived components, using a scatterplot to illustrate the variation within and between groups, while loadings were used to show variables that have potential discriminating impact on sample clustering in addition to explaining variation.

Immunocytochemical localisation of TGFβ. Serial sections (3-µm-thick) of paraffin wax-embedded cultures were obtained. Following standard dewaxing and rehydration procedures, antigen retrieval in freshly prepared 0.1 M Tris, 5% urea buffer (pH 9.5) at 95˚C for 10 min in an oven was conducted. This was followed by three 5-min washes in distilled water, and incubation in 1% bovine serum albumin in PBS-Tween for 30 min at room temperature for concurrent blocking of non-specific binding sites and permeabilisation. TGFβ was localised using a polyclonal rabbit antibody to TGFβ (Abcam) (detecting all TGFβ isoforms) at a concentration of 1:500, in PBS-Tween at 4˚C. Following overnight incubation, sections were washed in PBS (3 × 5 min) and incubated for 2 h at room temperature with Alexa Fluor 488 anti-rabbit (Life Technologies, Johannesburg, South Africa) secondary antibody diluted to a concentration of 1:1,000 in 1% bovine serum albumin

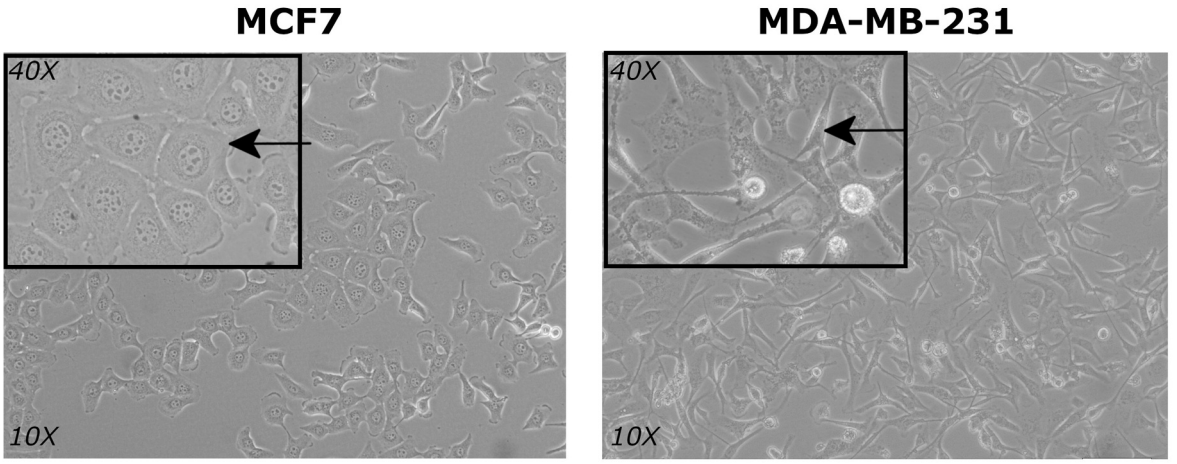

Figure 1. *Representative photomicrographs showing typical 2D morphology of breast cancer cells. Left: MCF-7 cells forming cell-cell adhesions (10× magnification) with fusiform morphology. More established cell masses with cells showing a polygonal morphology and cobble-stone appearance (arrow) (inset, 40× magnification). Right: MDA-MB-231 cells forming networks (10× magnification), with a typical spindle-shaped morphology (arrow).*

in PBS. Subsequently, sections were washed in PBS (3×5 min). Nuclei were counterstained with 4',6-diamidino-2-phenylindole diluted to 1: 50,000 in PBS for 5 min followed by two washes in PBS (5 min/wash). Sections were mounted in Fluoromount (Sigma-Aldrich, F4680) and stored at 4˚C until viewing.

Images were obtained using an Olympus iX51 inverted fluorescence microscope with CellSens software. The following filters were used: U-MWIB2 (Alexa Fluor 488), U-MIY2 (Alexa Fluor 594) and U-MNU2 (DAPI). Exposure time was set at 200 ms for all images. All images were obtained at 20× magnification and enlarged if need be using the software application, with scale bars automatically being adjusted. Plates were generated using GIMP 2.10.12.

Results and Discussion

Morphological characterisation of luminal and basal 3D culture models. Three-dimensional culture systems are accepted as physiologically relevant models, which more accurately capture the *in vivo* microenvironment than their 2D, monolayer counterparts. By providing extracellular matrix components and spatial configuration to enable cell–cell and cell–ECM interaction, cells are able to adopt a phenotype and behaviour more closely aligned with those of patient tumour samples (39-41). In 2D monolayer cultures, individual MCF-7 cells were noted to have a fusiform morphology while forming cell–cell associations (Figure 1); however, cells assumed a typical polygonal morphology when closely associated in a cell mass, adopting a cobble-stone epithelial-like appearance for which this cell line is known (30, 42). MDA-MB-231 cells, conversely, were predominantly spindle-shaped, reflecting their more aggressive, mesenchymal-like phenotype (42), and maintained this morphology while establishing cell–cell associations as irregular networks (Figure 1).

In the 3D culture system, cell lines required up to 48 h to spread within the GFRM. A limitation of this system is the visualisation of the depth of the 3D model in live, unstained cultures. However, it is apparent that in the control BC group of MCF-7 cells in the LPM retained their polygonal morphology but formed masses with indistinguishable cell borders (Figure 2), reminiscent of more well-differentiated tumours (42-44). MDA-MB-231 cells required a greater duration of time to establish themselves. Nevertheless, in the indicated BC control groups, these cells retained their spindle-shaped morphology forming networks when in close association. Moreover, this morphology reflects invadopia-like structures, the generation of which are MMP-dependent (45). Further evidence for MMP effects were noted during harvesting of cultures where the GFRM viscosity in groups containing lymphocyte populations (albeit to a lesser extent in the T_{REG}-BC control culture group), was considerably reduced and indicated that the GFRM had undergone remodelling. However, this is postulated not to be solely due to immune cell migration, but rather to the effects immune mediation had on cell–cell associations in both models. In the experimental culture groups in which both lymphocyte populations were present, a major disruption in the formation of either MCF-7 cell masses or MDA-MB-231 cell networks was noted. A similar phenomenon was identified under NK cell influence, but less evident under T_{REG} lymphocyte-mediation alone (Figure 2). We postulate that in this scenario, breast cancer cells condition lymphocyte populations to facilitate, rather than impede, tumour progression. This concept is further

alluded to by our findings of cytoplasmic and extracellular expression of TGFβ.

TGFβ immunolocalization - master controller. TGFβ is the master controller of EMT in established breast tumours, whereby it facilitates the expression of a more invasive phenotype in preparation for metastasis (25, 26). The results obtained in this study affirm the constitutive expression of TGFβ in MCF-7 and MDA-MB-231 cell lines as shown by other studies (46-48). Analysis of TGFβ isoform mRNA expression indicates that while MCF-7 and MDA-MB-231 cells exhibit similar levels of TGF-β1, TGFβ2 expression is notably higher in MDA-MB-231 cells, and TGFβ3 expression higher in MCF-7 cells (48). Clinically expression of both TGFβ1 and -3 isoforms are associated with tumour aggressiveness (26). In this study, a pan TGFβ antibody was however, used to ascertain overall TGFβ expression.

In our 3D culture models, cytoplasmic TGFβ expression was predominantly of intermediate intensity in most culture groups (Figure 3). Cytoplasmic localisation of TGFβ reflects its production as a latent protein with storage in the cytoplasmic compartment (48). While several cells in each culture group in the LPCM, exhibited both cytoplasmic and perinuclear TGFβ expression, cells in the experimental culture group demonstrated attenuated cytoplasmic expression with the restriction of TGFβ to the perinuclear region. The experimental group in the BPM exhibited a similar pattern of expression albeit to a lesser extent. Perinuclear TGFβ expression is associated with a higher rate of biosynthesis (48, 49), and may thus reflect an increase in invasive potential.

The LPM experimental culture group also displayed extracellular TGFβ expression that was not readily discernible in the BPM experimental culture group. TGFβ stored in the cytoplasmic compartment can be released into the extracellular matrix where it is activated by proteases including MMP9 and MMP2 (50, 51). TGFβ in turn, *via* autocrine and paracrine actions, induces increased MMP expression, resulting in a positive feedback loop necessary for migration (48, 52), which in luminal phenotype tumours may also be associated with loss of oestrogen receptor-α expression and associated increased invasive potential (30, 53, 54). We thus suggest that luminal phenotype tumours may employ an extracellular, soluble form of TGFβ not only for enhancing their own limited capacity for migration, but also for mediating T_{REG} lymphocyte suppression of NK cell function (27, 28).

In this study, NK cells and T_{REG} lymphocytes were activated with IL2 for a short period. Such activation has been linked with MMP9 secretion and enhanced migratory ability (55). Degradation of the extracellular matrix, which is a necessary component of migration, is reflected in the present study by the reduction in viscosity of the GFRM,

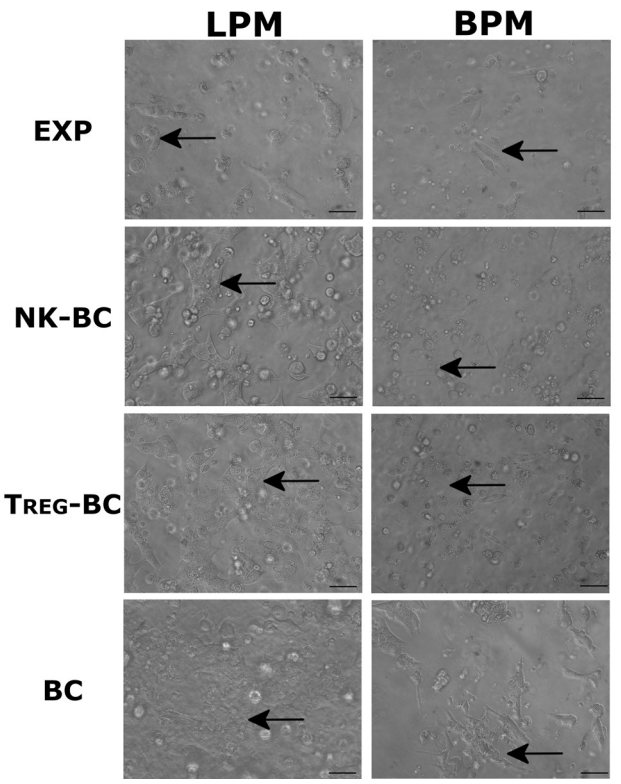

Figure 2. *Representative photomicrographs showing the established 3D morphology within a 72-h period. In the luminal phenotype model (LPM), MCF-7 cells maintained their more polygonal epithelial-like morphology, while in the basal phenotype model (BPM), MDA-MB-231 cells maintained a mesenchymal-like phenotype. In the experimental systems (EXP), with natural killer (NK) cells and T-regulatory (T_{REG}) lymphocytes, breast cancer cells were sparse and failed to generate the masses (LPM) or network pattern (BPM) when cancer cells were cultured alone (BC). Scale bar indicates 50 μm.*

particularly in the presence of lymphocytes in both models. We propose this is associated with an increase in MMP production facilitated by TGFβ secretion, also identified in the LPM NK-BC culture group and BPM NK-BC culture groups. TGFβ expression was more evident in the T_{REG}-BC culture group. In addition to its role in facilitating T_{REG}-mediated immunosuppression (27-29), sustained autocrine and paracrine TGFβ signalling is essential for maintenance of EMT and the generation of clonal stem cell populations in breast tumours (25, 26, 56).

Specifically, in basal phenotype tumours, represented in this study by the MDA-MB-231 cell line, high TGFβ1 and TGFβ2 expression is associated with malignancy (48). Luminal phenotype tumours, represented in this study by MCF-7 cells, are traditionally, weakly metastatic. Our results may thus reflect the induction of tumour mechanisms aimed not only at evading NK cell insult, but also at increasing the

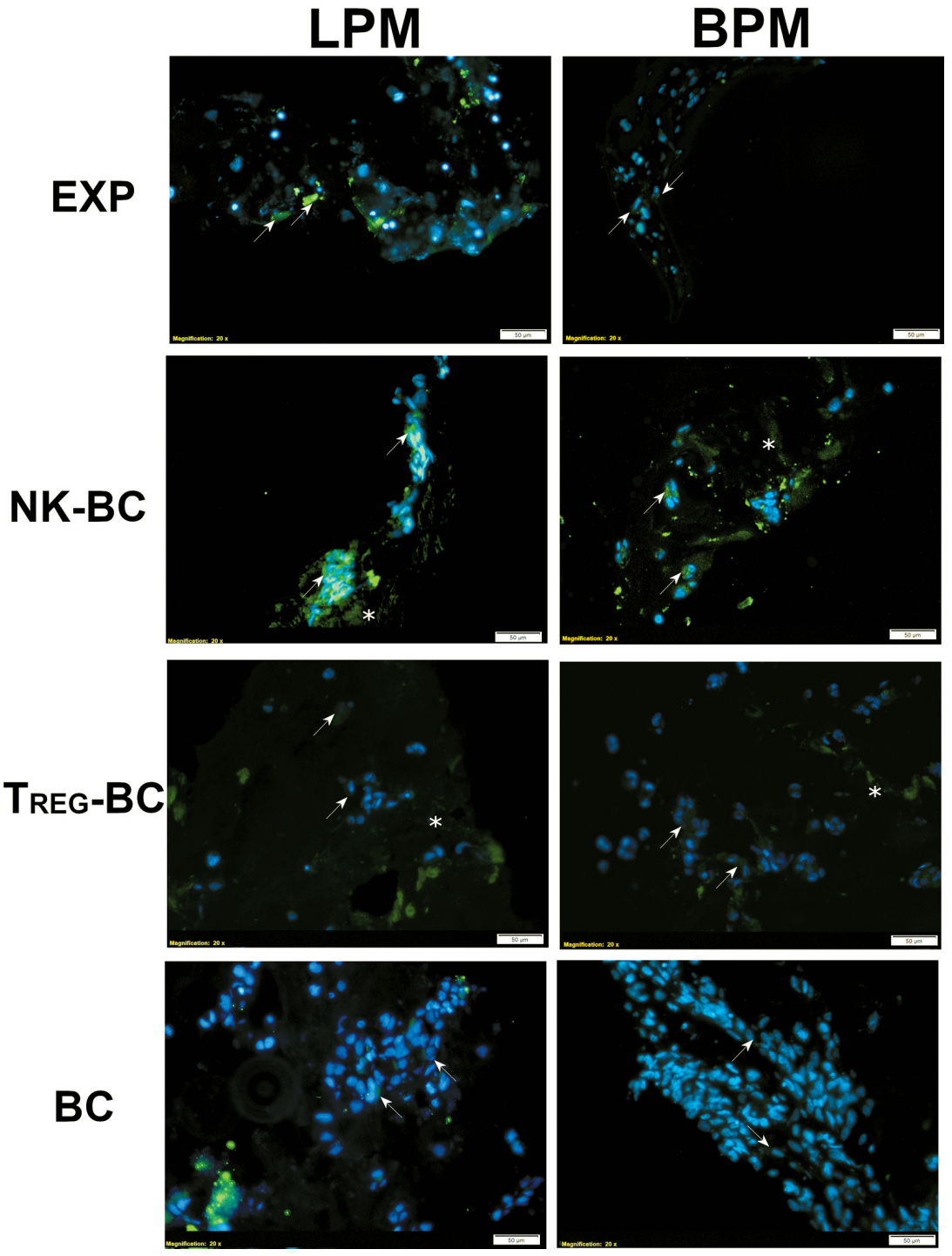

Figure 3. *Representative photomicrographs of immunolocalisation of transforming growth factor-β (TGFβ). In the luminal phenotype model (LPM), TGFβ expression (green) of intermediate intensity localised primarily to the cytoplasm (arrows), with some areas of high intensity perinuclear expression noted (4',6-diamidino-2-phenylindole, blue nuclear stain). Where natural killer cells (NK) were cultured with breast cancer cells (BC), cells exhibited higher intensity TGFβ expression in the extracellular matrix (ECM) (asterisk). TGFβ expression in the experimental culture group was considerably attenuated, with primarily perinuclear (arrows) and ECM (asterisk) expression. In the basal phenotype model (BPM), diffuse TGFβ expression of intermediate intensity localised primarily to the cytoplasm, with some cells exhibiting high-intensity perinuclear expression (arrows). TGFβ expression was noted to extend into the ECM (asterisk) primarily in the BC and T-regulatory lymphocyte and breast cancer cell (T_{REG}-BC) culture groups. Greater cytoplasmic expression (arrows) was noted in the experimental culture group compared to the same in the LPM.*

invasive potential of tumour cells themselves. These results echo morphological assessment (Figure 2) where, in comparison with controls which lacked immune cell mediation, both models showed a less differentiated phenotype (42, 44), particularly in those culture groups that contained T_{REG} lymphocytes. This failure to produce masses in the case of MCF-7 cells, or large networks in the case of MDA-MB-231 cells, taken together with TGFβ expression, is indicative of the acquisition of a more malignant phenotype.

Cytokine analysis – tumour cells mediate inflammation. The process of EMT, migration and invasion are all affected, to a large degree, by cytokines. Investigations to determine the interactions between multiple cytokines call for more sophisticated exploratory analyses to assess synergistic and antagonistic relationships (33, 35, 57, 58). A limitation of fluorescence multiplex assays is that median fluorescence intensities (observed fluorescence) are corrected for background and are assigned concentration values using a standard curve (sigmoidal or logistic curves generated from known analyte concentrations), which inevitably results in signal loss in lieu of gaining relative concentration data (33). Moreover, since multiple analytes are assessed per sample, a dilution series catering specifically to each cytokine of interest is not possible – some resulting concentrations may thus be lower or higher than the respective lower limit or upper limit of quantitation per analyte in relation to the standard curve (33). Data assigned as OOR are typically subject to a variety of imputation methods, including mean value substitution, extrapolation or maximum likelihood estimation (34, 35), or regarded as a non-detection. While these mathematical censoring methods may approximate actual values, there remains the likelihood of losing entire variables (cytokines of interest) to the OOR phenomenon (33). The very absence of such cytokines may actually have biological meaning but cannot be analysed using traditional statistical methods, highlighting the importance of finding patterns and relationships in cytokine studies as opposed to the use of concentration data alone.

In this study, censoring of concentration data resulted in loss of testable variables and thus the inability to analyse several cytokines by traditional statistical analysis (Table I). The dataset was affected by non-detections (OOR) in IL1β and IL12 across all groups in both models; and IL2, IFNγ, TNFα across all groups in the LPM due to being out of range or having fewer than 50% datapoint entries. A similar scenario was obtained for CLCL2 in the experimental LPM, and CXCL8 in the experimental BPM (Table I). This is not an unusual occurrence in cytokine analyses (33), and due to these non-detections in the luminal phenotype model, it was not possible to perform traditional statistical analysis. However, the very presence of these cytokines in only the

Table I. *Averages of analyte concentrations (pg/ml) ±standard deviation. Comparison of luminal phenotype model (LPM) and basal phenotype model (BPM) matched culture group cytokine release.*

	BC			TREG-BC			NK-BC			Experimental		
	LPM	BPM	*p*-Value*	LPM	BPM	*p*-Value*	LPM	BPM	*p*-Value*	LPM	BPM	*p*-Value*
IL1β	(<OOR)	(<OOR)	N/A	(<OOR)	(<OOR)	N/A	(<OOR)	(<OOR)	N/A	(<OOR)	(<OOR)	N/A
IL2	(<50%)	4.15±0.83	N/A	(<OOR)	4.3±1.1	N/A	(<OOR)	3.4±1.8	N/A	(<50%)	4.3±1.3	N/A
IL6	3997.9±3038	5856±4118	0.41358	418.1±690	10896.6±4965	0.00133	21.3±8.7	8250.1±5141	0.00066	21.3±8.78	8250±5141	**0.00066**
IL12	(<OOR)	(<OOR)	N/A	(<OOR)	(<OOR)	N/A	(<OOR)	(<OOR)	N/A	(<OOR)	(<OOR)	N/A
IFNγ	(<50%)	123.7±21.4	N/A	(<50%)	121.6±23.5	N/A	(<OOR)	117.4±7.5	N/A	(<50%)	147.6±12.3	N/A
TNFα	(<50%)	24.7±14.6	N/A	(<OOR)	16±1.4	N/A	(<OOR)	16.4±12.1	N/A	(<50%)	22.7±3.2	N/A
CCL2	(<OOR)	44.9±29.6	N/A	(<50%)	68.6±11.4	N/A	648.4±483	153.2±112	0.14185	(<50%)	131.4±67	N/A
CCL4	2.21±0.06	3.49±0.94	**0.00066**	2.5±0.36	5.2±1.14	**0.00066**	27.5±37.3	26.1±32.2	0.75457	82.4±137	9.9±1.89	0.34498
CXCL8	2051.4±3680	6992±6966	0.08125	334.6±512	4027.9±3382	**0.0199**	455.8±556	4198.3±739	**0.00066**	2325.2±4920	(<50%)	N/A

IL1β/2/6/12: Interleukin-1β/-2/-6/-12; CCL2/4: chemokine C-C motif ligand 2/4; CXCL8: chemokine C-X-C motif ligand 8; IFNγ: interferon-γ; TNFα: tumor necrosis factor-α; ORR: data out of range; <50%: <50% data points available; BC: Control group of breast cancer cells only; T_{REG}-BC: control group with T_{REG} lymphocytes and breast cancer cells; NK-BC: control group with NK cells and breast cancer cells; Experimental group with T_{REG} lymphocytes, NK cells and breast cancer cells. *p*-values <0.05 reflected in bold. N/A indicates calculation could not be performed. *Two-sided test.

basal phenotype model highlights their importance in distinguishing the inflammatory nature of this phenotype.

We first determined whether matched culture groups differentially released cytokines depending on the breast cancer cell phenotype (LPM compared to BPM), using only complete responses (*i.e.* cytokines that were not detectable in any culture group were excluded) (Table I). Our results show that basal phenotype cells produced higher levels of all cytokines detected, with significantly higher levels of CCL4 ($p<0.05$); taking into account that the dataset of the BC group was affected by non-detections in IL2, IFNγ, TNFα and CCL2 variables (<OOR or <50%) in the LPM. Thus, while these cytokines were unable to be analysed for statistical significance, their presence in the BPM highlights the pro-inflammatory nature of the induced microenvironment. As expected, luminal phenotype, hormone-dependent breast cancer cells secreted less IL6 than the more aggressive, basal phenotype, hormone-independent cell line (1, 19). The hormone receptor status of breast tumours has been shown by several studies to be related to the cytokine profile present (6, 58-62); however, our results supplement this information by showing that immune mediation alters the cytokine landscape. Specifically, under T_{REG} immune mediation, detectable IL6 was significantly reduced in the LPM compared to stable expression in the BPM. This suggests depletion of this cytokine by T_{REG} lymphocytes, reflecting further activation of this immune cell subset (15).

Table II. *Loadings from principal component (PC) analysis of all cytokines conducted on all culture groups within the luminal phenotype model (LPM) and basal phenotype model (BPM). Three components (PC1, PC2, PC3) were derived, cumulatively explaining >90% of the total variance. Loadings indicated in bold show which cytokines had a high contribution to each component.*

Cytokines	Loadings of derived principal components		
	PC1	PC2	PC3
IL6	**0.58071**	–0.01268	**–0.59803**
CCL2	**0.40081**	–0.04873	**0.74968**
CXCL8	0.18293	**0.96965**	0.13191
CCL4	0.022001	0.053691	0.24947
IFNγ	**0.56719**	–0.10217	0.023207
TNFα	0.34009	–0.07912	0.006184
IL2	0.17557	–0.04873	0.01093
IL1β	–	–	–
IL12	–	–	–
Eigenvalue	3.04758	1.5934	1.02741
Percentage variance explained	49.042	25.641	16.533
Total percentage variance explained		**91.216**	

IL1β/2/6/12: Interleukin-1β/-2/-6/-12; CCL2/4: chemokine C-C motif ligand 2/4; CXCL8: chemokine C-X-C motif ligand 8; IFNγ: interferon-γ; TNFα: tumor necrosis factor-α.

Additionally, the results show that although the MCF-7 cell line is weakly metastatic (63), compared to the basal phenotype cell line, MCF-7 cells may have subverted T_{REG} lymphocytes to encourage their invasive potential. Basal phenotype cancers, represented in this study by the MDA-MB-231 cell line, are associated with cytokines reminiscent of a more aggressive phenotype (1, 64). In the present study, under T_{REG} lymphocyte mediation, all available cytokines (IL6, CCL4 and CXCL8) were significantly higher in the BPM than the LPM. This echoes studies in which T_{REG} lymphocytes are shown to promote tumour progression (3, 12, 13).

Under NK cell mediation alone (NK-BC), again several cytokines were not able to be assessed; however, of the available cytokines, the outcome revealed significantly higher IL6 and CXCL8 levels in the BPM ($p<0.05$) compared to the LPM (Table I). In the experimental group, it was only possible to assess IL6 and CCL4, with the former raised ($p<0.05$) in the BPM. Further investigation within each model, revealed that IL6 showed considerable variation in the LPM, being reduced in all culture groups exposed to immune mediation and significantly so ($p=0.046$) under NK cell mediation (NK-BC) (Figure 4). CCL4 was also significantly increased in the experimental group compared to the control BC group ($p=0.0003$) and to the T_{REG}-BC group ($p=0.0036$) in the LPM (Figure 4). In the NK-BC group, CCL4 was significantly raised compared to the BC group ($p=0.006$). Similarly, in the basal phenotype model, CCL4 was also noted to be raised in the experimental group compared to BC control ($p=0.0019$), with considerable variation in the NK-BC group. Notably IL6, both tumour-derived and T_{REG} lymphocyte-secreted, is also associated with the suppression of NK cell function (6), which when coupled with consideration of TGFβ expression (Figure 3) (27-29), further affirms our contention that both tumour models have a dampened NK cell response, evidently more so in the basal phenotype model. In both models, CCL4 was significantly raised in the experimental groups which, since this chemokine is associated with promoting breast cancer metastasis (65), supports our results indicating heightened tumour invasive capacity. Nevertheless, we were limited by traditional analysis in that it was not possible to assess some variables due to the absence of concentration data in the LPM (Table I); as such, we included exploratory analysis to additionally investigate cytokine patterns that would reflect the complexity of cytokine networks and resulting multifaceted functions (1, 6, 17, 22).

Since cytokines are commonly associated or correlated with each other (as also found in this study), using a regression model would introduce effects of collinearity (66). To circumvent this problem, principal component analysis was used, reducing variables (based on a correlation matrix) into components accounting for the majority of the variance

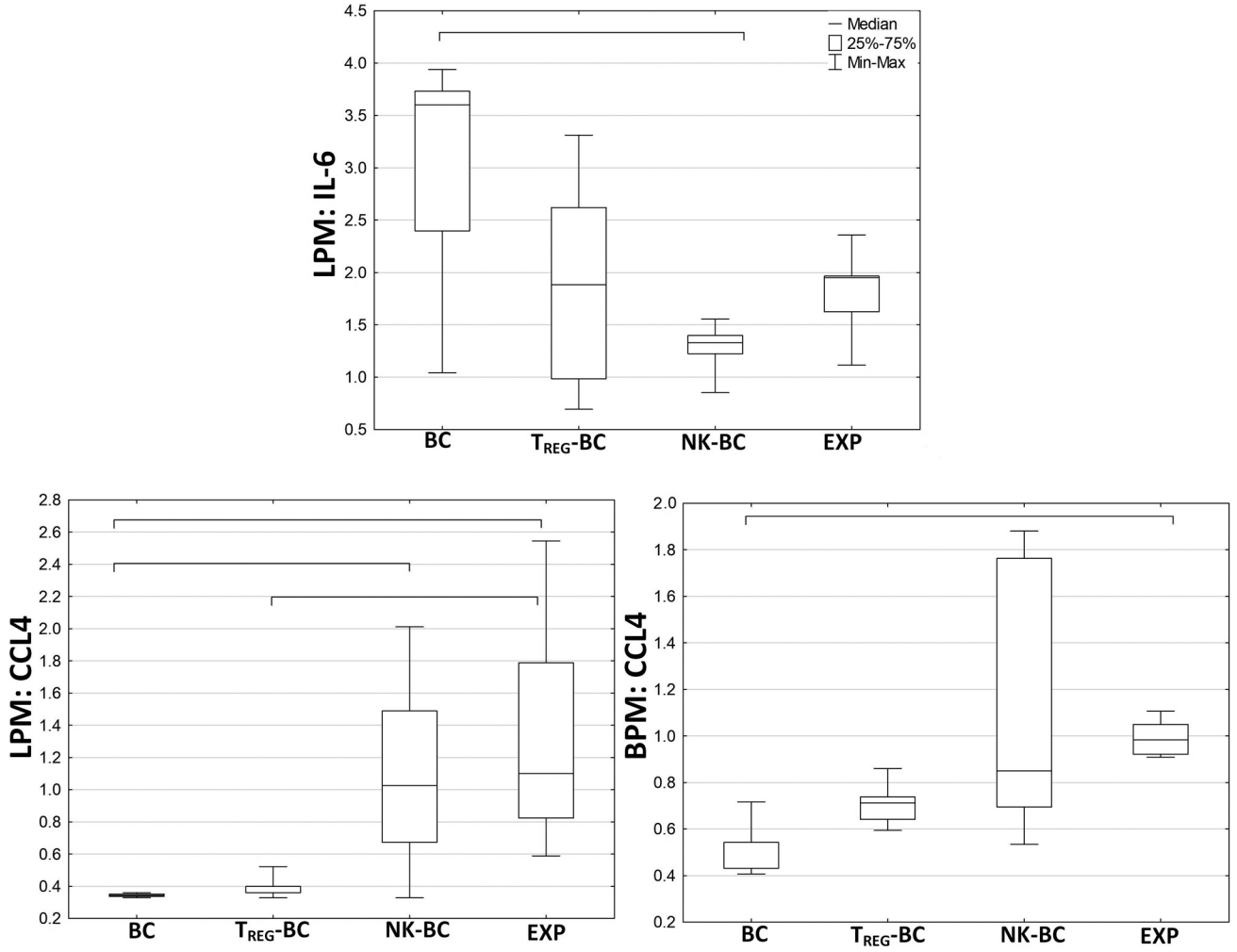

Figure 4. *Box and whisker plots for visual representation of significant (p<0.05 designated by bars) immune mediation of cytokines within the luminal phenotype model (LPM) and basal phenotype model (BPM).*

in the dataset and identifying patterns of expression (35, 66, 67). Three principal components were derived, accounting for 91.216% of the variance (Table II). Factor loadings indicated that IL6, CCL2, CXCL8 and IFNγ had important contributions to the derived components, and additionally can be used to differentiate between culture groups, and between the models themselves. The scatter plot shown in Figure 5 indicates model delineation along both axes. IL6 and CCL2 had strong, positive contributions to PC1 and PC3 (Table II). PC1 describes primarily, discrimination between the luminal phenotype model and basal phenotype model (Figure 5). The addition of IFNγ to PC1 aided in better discrimination between the models. Furthermore, CCL2 and IL6 on PC3 are seen to accommodate the separation of the NK-BC culture groups within the LPM, with CXCL8 on PC2 separating LPM and BPM breast cancer cell controls (BC). Notably close association of T_{REG}-BC and BC culture groups in the models are identified indicating marked similarity in induced cytokine response. This is related to the expression of IL6 and CCL2 (PC1, BPM; PC3, LPM).

Taken together, the principal component analysis supports differences in cytokine concentrations where IL6 and CXCL8 differed between culture groups within and between the models, whereas CCL2 and IFNγ were detectable only in selected culture groups in the LPM but present throughout the BPM and did thus not undergo traditional statistical analysis. In contrast CCL4 was not identified as a strong contributor to the derived PCs. Principal components analysis supplemented the data derived from traditional analysis alone, shedding further light on the importance of selected cytokines in understanding the response of luminal and basal phenotype cells to immune mediation.

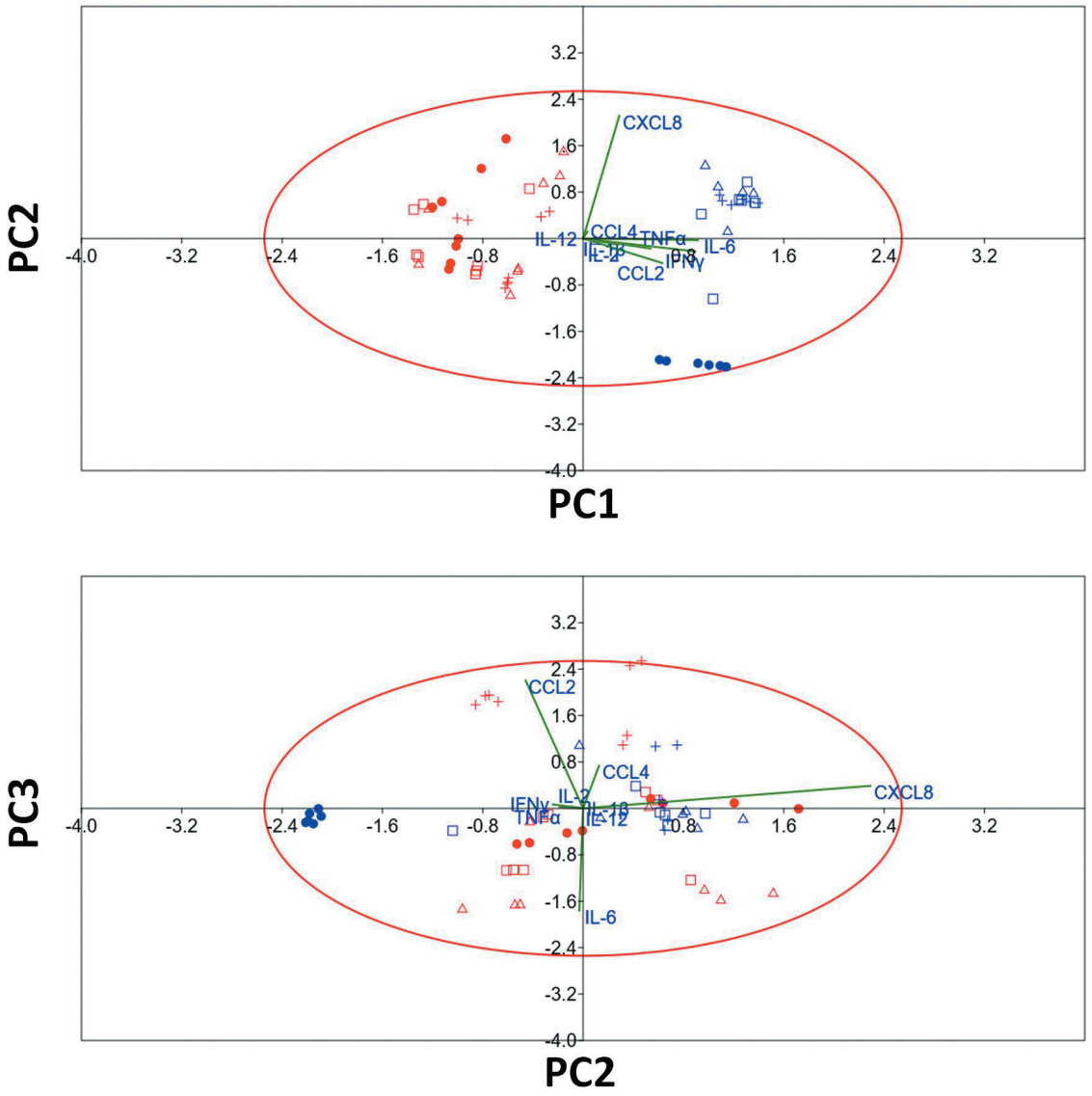

Figure 5. *Scatterplot of principal component 1, 2 and 3 loadings with cases and cytokine biplot encased in a 95% confidence ellipse. Key: Luminal phenotype model (LPM) in red, and basal phenotype model (BPM) in blue; Culture groups: triangle: breast cancer (BC) cells alone; square: T-regulatory (T_{REG}) lymphocytes and BC cells; plus symbol: natural killer cells (NK) and BC cells; shaded circle: experimental with T_{REG} lymphocytes, NK cells and BC cells. Interleukin-6 (IL6) and chemokine C-C motif ligand 2 (CCL2) retained influence on PC1, together with interferon-γ (IFNγ). PC2 was highly influenced by chemokine C-X-C motif ligand 8 (CXCL8). Negative loading of IL6 and positive loading of CCL2 on PC3 differentiated culture groups.*

The results of this study identified that an IL6-chemokine axis is primarily responsible for differentiating breast cancer models. While this was strongly associated with phenotype in our system, further investigation into the response shows that under immune mediation, luminal and basal phenotype breast cancer cells respond differentially, with the basal phenotype model generating a more inflammatory microenvironment. This complements the findings of several clinical studies (1, 6, 60, 61) but by deconstructing the *in vivo* microenvironment is able to better delineate the roles of specific immune subsets. A potential limitation of this study is the absence of T_{REG} lymphocytes and NK cells cultured without tumour cells; however, we sought to recreate a tumour microenvironment where these cells would not function in isolation, but rather where reciprocal interactions could be investigated.

In 3D systems and *in vivo*, IL6 is strongly associated with breast tumour growth and progression (18). Notably the pro-

tumorigenic function of IL6, mediated by glycoprotein (GP)-130 activation of signalling pathways, results not only in the induction of survival mechanisms but also in the increase in oestradiol-17β hydroxysteroid dehydrogenase type I which is necessary for the conversion of estrone to oestradiol, and is thus particularly beneficial for luminal phenotype tumour progression (1). The dominant association of IL6 with the chemokines highlights the role of this pro-inflammatory cytokine in initiating leukocyte infiltration (68) and speaks to its role in modulating tumour-infiltrating immune cell function (18). In the present study this is evident by the depletion of this cytokine during immune mediation in the luminal phenotype model, we postulate as a pro-tumorigenic strategy. In our culture system, IL6 secretion in the basal phenotype model increased significantly compared to the luminal phenotype model under immune mediation, implying a tumour cell response to immune cell subsets. It has, however, been hypothesized that immune cells in apposition with cancer cells may be equally capable of eliciting large amounts of IL6 for tumour progression (69). IL6 has further been linked with shifting the inflammatory response from innate immunity to adaptive immune control during infections (22, 70). A similar phenomenon has been identified with IFNγ, which while critical for activation of NK cell migration and antitumour responses, also mediates induction of adaptive immunity to elicit pro-tumorigenic functions (6, 71). Specifically, IFNγ has also been implicated in the suppression of NK cell function *via* the up-regulation of T_{REG} lymphocyte activity (72), and the up-regulation of tumour-associated major histocompatability complex (MHC)-I molecules, thereby protecting tumour cells from NK cell-mediated lysis (71). This capacity is also postulated to be driven by TGFβ (73). Additionally, IFNγ is linked with preventing tumour infiltration of innate immune cells (6). Functionally, this phenomenon might describe the accumulation of T_{REG} lymphocytes and the scarcity of NK cells in TIL populations (4, 10), which reflect the supremacy of adaptive immune mechanisms in advanced breast cancer.

Studies show that abrogation of IL6 signalling in hormone-dependent tumours reduces tumour growth and aggressiveness, highlighting the potential for anti-IL6-based therapies (18, 19). Our results indicate that hormone-independent tumours may be similarly affected. This is further highlighted by findings that IL6, in association with TNFα, is implicated in facilitating adhesion of basal phenotype MDA-MB-231 cells during extravasation into the vascular supply (62). Considering the secretion of pro-inflammatory T-helper type 1 cytokines IFNγ, TNFα and IL2, the results are suggestive of tumour progression (19), particularly in the basal phenotype model, echoing clinical assessments (74).

The IL6–chemokine axis was also illustrated with CCL4 expression increasing significantly under immune mediation in both models, and the importance of CCL2 and CXCL8 described by exploratory analysis. Both CCL2 and CCL4 are associated with poor prognosis in patients with breast cancer, with increased levels linked to metastatic spread and with limiting the ability of the immune response to eradicate tumour cells (22, 65, 75). Together CCL4, CCL2 and CXCL8 show high efficacy as being pro-angiogenic factors and are also implicated in tissue remodelling *via* MMP induction (21, 22), which in turn is necessary for the infiltration of immune cells and thus maintenance of the chronic inflammatory microenvironment. In this study, CXCL8 showed considerable power in separating basal and luminal phenotype models, while accommodating separation of the culture groups containing NK cells. CCL2 is further suggested to act synergistically with IL6 (22); however, abrogation of CCL2 in MDA-MB-231 cell xenografts, while resulting in inhibition of tumour progression, was not found to affect IL6 expression (23), stressing the complexity of cytokine signalling pathways. IL6 is also able to engage other pathways as evidenced by its cross-talk with TGFβ which in turn facilitates tumour progression (76, 77), again highlighting the complexity of cytokine signalling in mediating cell–cell interactions.

Conclusion

Despite the utility of 3D models in recapitulating the tumour microenvironment, there remain a paucity of studies investigating immune mediation of tumour processes. This study used a 3D model to assess the response of luminal and basal phenotype tumour cells to immune mediation. Our results indicate that both tumour phenotypes were able to modulate T_{REG} lymphocyte and NK cell function to facilitate, rather than impede, tumour progression. Traditional and exploratory statistical analysis identified an IL6–chemokine axis, which, together with TGFβ expression which was visualised both in the cytoplasmic and extracellular space, are proposed as drivers of tumour progression. While this was strongly associated with phenotype in our system, further investigation determined that luminal and basal phenotype breast cancer cells respond differentially to immune mediation, with the basal phenotype model generating a more inflammatory microenvironment. In addition, TGFβ, primarily in the luminal phenotype model, and coupled with IFNγ in the basal phenotype model, may reflect the induction of tumour mechanisms aimed at evading NK cell insult, under control of the tumour cells themselves with contributions from T_{REG} lymphocytes. Under the direction of the IL6–chemokine axis, tumour cells were able to subvert lymphocyte subsets to disrupt the formation of tumour cell masses and networks associated with more well-differentiated phenotypes, and to promote a more invasive phenotype. This was also reflected by a

change in the viscosity of the extracellular matrix. Further investigation of these parameters will be necessary to describe tumour-immune cell interactions.

Conflicts of Interest

The Authors report no conflicts of interest.

Authors' Contributions

TNA was responsible for the concept and design of the study, acquisition of data, data analysis and interpretation, and drafting of the article. RD and GC contributed to the design, data interpretation and critical revision of the article.

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Data availability

The data will be made available on request to the corresponding Author and by application to the Human Research Ethics Committee, University of the Witwatersrand.

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